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Introduction

The serine/threonine kinase Akt has been implicated in the initiation and/or progression of breast carcinomas (1) (2). The cellular and molecular events that are altered upon the constitutive activation of Akt are not completely understood. Akt could play a role in initiating and/or contributing to the progression of breast cancer by phosphorylating key intermediates in growth signaling pathways. Alternatively, Akt could prevent apoptosis by phosphorylating key components of an apoptosis pathway. To further our understanding of how Akt initiates and/or promotes the progression of breast carcinomas, we used molecular, cellular and biochemical approaches to identify and characterize regulators and effectors of Akt.

Work Accomplished to Date

Summary

We proposed to identify effectors of Akt (Task 1). We identified Brn-1, a POU domain transcription factor (June 1999 annual report), and the B-Raf serine/threonine kinase (June 2000 annual report) as downstream effectors (targets) of Akt. Our research on the regulation of B-Raf by Akt was published in the *Journal of Biological Chemistry* [(3), see appendix]. While studying the regulation of B-Raf by Akt, we found that Akt negatively regulates the activity and stability of the Elk-1 transcription factor. Our efforts in the last year have been directed towards understanding the molecular and biochemical basis of this regulation of Elk by Akt and this work is described in greater detail below.

We also proposed to identify and characterize regulators of Akt (Task 1). The lipids synthesized by phosphatidylinositol 3-kinase (PI3K) activate the enzymatic activity of Akt (4). Upstream activators of PI3K include members of the Ras and Rho family of small GTPases (5) (6). Clearly, events that regulate the activity and/or the subcellular localization of Ras and Rho will play a critical role in regulating PI3K activity and, consequently, Akt activation. Events that promote the GTP bound state of Ras and Rho or events that promote their membrane localization will activate PI3K and, subsequently, Akt. We have found that PRA1 (prenylated Rab acceptor protein) binds both farnesylated and geranylgeranylated small GTPases, including Ras and Rho (7). We suggest that PRA1 acts as an escort protein for small GTPases by binding to and "solubilizing" the hydrophobic isoprenoid moieties of the small GTPases and thereby facilitates their trafficking through the endomembrane system. Our studies on the association of PRA1 with Ras and Rho have been accepted for publication in the *Journal of Biological Chemistry* [(7), see appendix]. Perhaps inhibitors directed against PRA1 may provide a means to pharmacologically intervene to inhibit tumorigenesis and metastasis involving the Ras/Rho/PI3K/Akt pathway.

Since the grant began in May of 1998, we completed Task 1, identification of regulators and effectors of Akt. We performed a yeast two-hybrid screen (Task 1, part a), sorted the library positives into classes (Task 1, part b), restriction mapped and sequenced library positives (Task 1, part c), characterized the interaction of Akt and its partners in vitro (Task 1, part d), performed in vitro kinase assays (Task 1, part e), and obtained full length clones of interacting proteins (Task 1, part f). We demonstrated that Akt uses multiple effector pathways, Task 2. We constructed plasmids for expressing the Akt interacting proteins in tissue culture cells (Task 2, part a) and examined changes in gene expression (Task 2, part b). We published two manuscripts (Task 2, part d) and have made substantial progress towards a third manuscript

(Figueroa, Taylor and Vojtek, "Akt targets the destruction of the Elk-1 transcription factor," in preparation).

Akt decreases the activity and stability of the Elk-1 transcription factor.

The Elk protein is a downstream target of the Ras pathway and is implicated in the activation of the c-fos immediate early gene (8) (9). In rat-1 fibroblasts and in the MCF7 breast cancer cell line, ectopic expression of Elk promotes cell death in response to calcium ionophore treatment (10). Elk may promote cell death by activating death inducing genes, such as Bad. Alternatively, since c-fos is associated with cell death in a number of different systems (11) (12) (13), Elk may promote cell death by activation of c-fos. We have found that Akt promotes the degradation of the Elk protein. Thus, Akt may promote cell survival, in part, by targeting Elk for degradation.

A. Akt expression blocks induction of Elk-1-dependent transcription.

We tested the effect of a constitutively active Akt on Elk-1-dependent transcription by assaying the activity of a Gal4-ElkC reporter. Gal4-ElkC contains the carboxy terminal domain of Elk-1, which includes the transactivation domain of Elk and the Erk phosphorylation sites, fused to the DNA binding domain of the yeast Gal4 protein. The transcriptional activity of Gal4-ElkC is dependent on phosphorylation of the Elk transactivation domain by Erk (8).

COS cells were transfected with expression vectors for Gal4-ElkC, Gal4-luciferase, pcDNA3 (vector control) and SuperMek (a constitutively active MEK in which the serines phosphorylated by Raf are changed to aspartic acids), alone or together with myr-AKT (a membrane localized, constitutively active version of Akt). Akt expression blocked the induction of the Elk-1 reporter by superMEK, Figure 1. Thus, although Akt can interfere with Ras signaling by directly phosphorylating Raf (3), the Ras pathway must also be targeted by Akt at an additional site, at or downstream of, MEK.

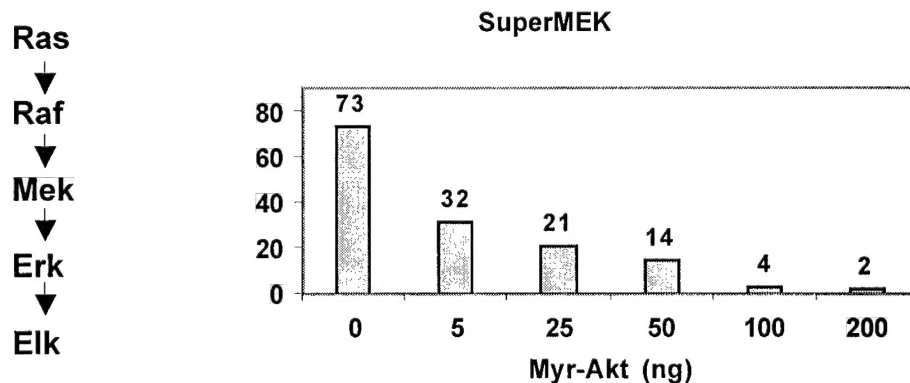


Figure 1. Akt expression blocks induction of Elk-1-dependent transcription activity by activated Mek. COS cells were transfected with expression vectors for Gal4-ElkC, Gal4-luciferase, SuperMek, and constitutively active Akt or not. The concentration of Akt expression vector ranged from 0 to 200 ng. Luciferase activity, assayed 36 hours after transfection, was normalized to a co-transfected β -galactosidase expression vector. Akt blocks the induction of Elk-1 dependent transcription activity by activated Mek in a dose dependent manner.

B. The Elk protein is decreased in cells transfected with constitutively active Akt.

HEK293 cells were transfected with pcDNA3-HA-Elk (full length Elk in pcDNA3 epitope tagged at the amino terminus with HA), pcDNA3-K-RasV12, and with and without pcDNA3-myr-HA-AKT. In each transfection, an expression vector for KSR, pcDNA3-HA-KSR, was introduced for an internal transfection control. Following SDS-PAGE, western blotting was used to detect Elk, Erk and Akt in the extracts. Because we have shown that Akt phosphorylates and negatively regulates B-Raf enzymatic activity, we expected that the phosphorylation of Elk by Erk would be blocked in the presence of Akt. To our surprise, the level of the Elk protein is greatly reduced in extracts prepared from cells transfected with myr-Akt, Figure 2.

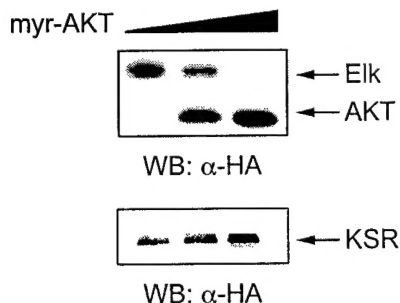


Figure 2. Akt reduces the level of Elk but not an internal control, KSR. Expression vectors for Elk and KSR were introduced into HEK293 cells without an Akt expression vector (lane 1) or with increasing concentrations of the Akt expression vector (lanes 2 and 3). Elk, Akt and KSR were detected by western blot analysis with an antibody directed against the HA epitope. HA-KSR is internal transfection control, which is expressed from the same promoter as Elk and Akt, human CMV.

There are four possible mechanisms for the decrease in Elk protein in the presence of myr-Akt. First, Akt could decrease transcription of Elk. However, we see a decrease in Elk protein when Elk is expressed under control of a heterologous promoter, the human cytomegalovirus promoter, and Akt does not dampen the expression off of this promoter under the conditions used in our assays. So, an effect of Akt on Elk transcription is unlikely. Second, Akt could promote degradation of the Elk RNA. This is possible with the caveat that an RNA sequence within the Elk coding region must mediate this event. Third, Akt could decrease the translation of Elk RNA. Usually, translational control elements are located within 5' and 3' untranslated regions, which our constructs do not contain. We think this model is unlikely. However, we cannot formally exclude the possibility that a sequence within the coding region of Elk is acting as a translational control element. Fourth, Akt could promote the degradation of the Elk protein. As described below, our recent data strongly suggest that this model is the most likely.

In mammalian cells, most intracellular protein degradation occurs via the proteasome, a multi-catalytic protease complex (14) (15). To determine whether the disappearance of Elk was mediated by the proteasome, we asked whether the Elk protein could be stabilized by the

proteasome inhibitor MG132. Cells were transfected with expression plasmids for HA-Elk, constitutively active Ras, and constitutively active Akt and then treated or not with MG132. Extracts were prepared and subjected to SDS-PAGE followed by western blotting with Elk-specific antibodies. As shown in Figure 3A, the Elk protein was stabilized in the presence of Akt upon the addition of MG132. Since MG132 can also inhibit calpains (14) (15), we determined whether the calpain inhibitor calpeptin could also stabilize Elk. Addition of calpeptin did not stabilize the Elk protein, Figure 3B. MG132 can also inhibit certain lysosomal cysteine proteases. To exclude a role for the lysosome in the disappearance of Elk, we will assess the stability of the Elk protein in the presence and absence of ammonium chloride (10 mM). However, lysosomal proteases are primarily involved in the degradation of membrane-associated proteins and proteins that have entered the cell by endocytosis whereas most intracellular protein degradation occurs via the proteasome (14) (15). Taken together, the results from our inhibitor studies strongly suggest that the disappearance of Elk in the presence of Akt is due to degradation of the Elk protein, likely via the proteasome.

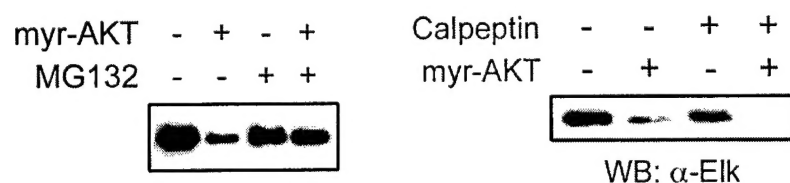


Figure 3. Elk is stabilized in the presence of the proteasome inhibitor MG132 but not the calpain inhibitor calpeptin.

We have recently implemented an inducible system for the activation of the Akt kinase (16). The hormone binding domain of the tamoxifen mutant estrogen receptor was fused to a constitutively active Akt; this Akt is refractory to upstream regulation: the PH domain is deleted and phosphorylatable serines are substituted with aspartic acids to mimic the phosphorylation events normally occurring at these residues (ERTM ΔPHDDAkt). An inducible kinase defective Akt was also constructed (ERTM ΔPHDDAkt K/D). Such fusions to the hormone binding domain create inactive proteins that become functional in minutes once hormone (4-OH tamoxifen) is added to the culture medium.

We examined the decrease in Elk protein after activation of the Akt fusion protein by the addition of hormone, Figure 4. We see a modest decrease in Elk protein within 20 minutes of the addition of hormone. A striking 13X decrease in Elk protein occurs between 20 and 40 minutes. The decrease in Elk protein is not seen when the cells express a catalytically inactive Akt, indicating that the kinase activity of Akt is required for the decrease in Elk protein. The decrease in Elk protein is biphasic. The initial response (3X decrease in protein levels during the first 20 minutes of hormone treatment) likely includes the time needed to release Akt from inactive complexes in the cytoplasm and translocate into the nucleus. The rapid and dramatic decrease in Elk protein levels 20 to 40 minutes after addition of tamoxifen, taken together with our data showing the stabilization of Elk protein by a proteasome inhibitor in the presence of Akt (Figure

3), are consistent with a model in which Akt promotes the degradation of the Elk protein. Pulse-chase analysis of Elk under these conditions is in progress.

Many regulatory proteins are targeted to the ubiquitin/proteasome pathway by regulated phosphorylation events. One possible mechanism for Elk degradation is that Akt activates a kinase that phosphorylates Elk and promotes its degradation. The inducible system described here will facilitate the phosphopeptide mapping of the Elk protein after Akt activation.

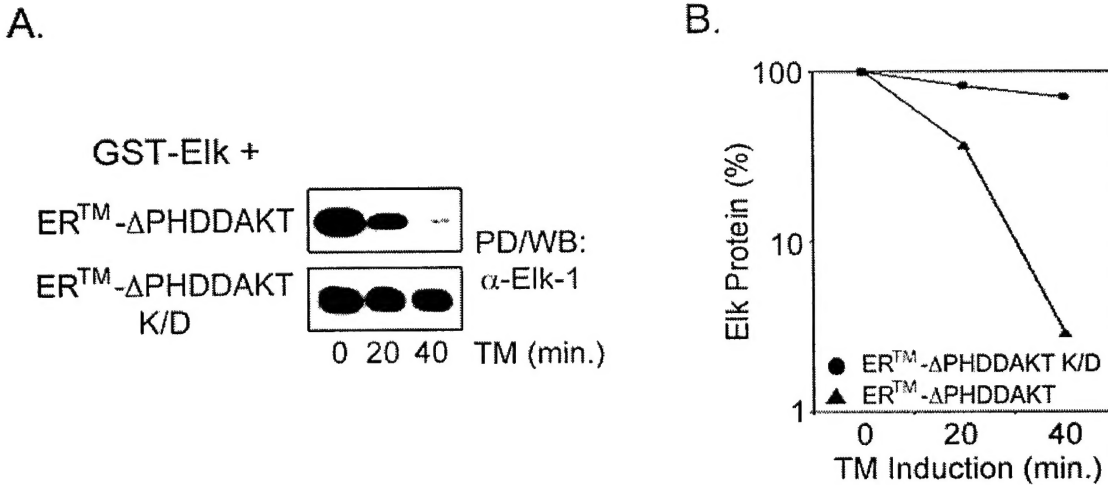


Figure 4. Elk protein levels rapidly decrease upon induction of a constitutively active Akt protein. HEK293 cells were transfected with expression constructs for GST-Elk and inducible Akt, either kinase active or inactive (K/D). At time 0 (18 hours after transfection), hormone was added and GST-Elk was harvested by precipitation with glutathione sepharose resin. Elk was detected by western blot analysis. The precipitated Elk shown in (A) was quantitated by densitometric analysis (B). Time 0 was set at 100. Data is plotted on a semilog scale.

Key Research Accomplishments

- Identified the Brn-1 transcription factor as an Akt interacting protein, using a yeast two-hybrid screen
 - Provided secondary evidence of complex formation between Akt and Brn-1
 - Demonstrated that S407 in Brn-1 is phosphorylated by Akt
 - Taken together, these results suggest that the activity of the Brn-1 transcription factor is likely to be regulated by Akt
-
- Demonstrated that Akt and B-Raf co-associate in vivo
 - Demonstrated that B-Raf is phosphorylated by Akt at multiple residues within its amino terminal domain (S364 and S428)
 - Demonstrated that alteration of the serine residues within the Akt consensus sites in B-Raf to alanine results in a progressive increase in enzymatic activity in vitro and in vivo
 - Expression of Akt inhibits EGF induced B-Raf activity and inhibition of Akt with LY294002 upregulates B-Raf activity, suggesting that Akt negatively regulates B-Raf in vivo
 - Taken together, these results show that the B-Raf kinase is negatively regulated by Akt in vivo and in vitro
-
- Demonstrated that Akt expression blocks induction of Elk-1-dependent transcription activity by activated Mek
 - Demonstrated that Akt reduces the level of Elk but not an internal control, KSR.
 - Elk is stabilized in the presence of the proteasome inhibitor MG132 but not the calpain inhibitor calpeptin, suggesting that Akt promotes the targeted destruction of the Elk protein via the proteasome
 - Showed that Elk protein levels rapidly decrease upon induction of a constitutively active Akt protein and that Akt kinase activity is required
 - Taken together, these results suggest that the Elk-1 transcription factor is negatively regulated by Akt

Reportable Outcomes

1. Bibliography of Publications

Guan, K-L., Figueroa, C., Brtva, T., Taylor, J., Barber, T., Zhu, T. and Vojtek, A. (2000). Negative regulation of the B-Raf serine/threonine kinase by Akt. *J. Biol. Chem.* **275**, 27354-59.

Figueroa, C., Taylor, J., and Vojtek, A. B. (2001). PRA1 is a receptor for prenylated small GTPases. *J. Biol. Chem.*, in press.

2. Funding Applied For Based on Work Supported by this Award
American Cancer Society Research Scholar Grant
Beginning July 2001

3. Degrees Obtained
Claudia Figueroa is expected to receive her Ph.D. in December 2001

4. List of Meeting Abstracts
Era of Hope Department of Defense Breast Cancer Research Program
Meeting, June 8-11, 200

5. List of Personnel Receiving Pay from DAMD17-1-98-8319
Anne B. Vojtek, Assistant Professor
Claudia Figueroa, Graduate Student
Jennifer Taylor, Research Associate
May Tsoi, Lab Aid
Tamara Kouskoulas, Undergraduate Work Study Student

Conclusions

Akt is involved in the initiation and/or progression of breast cancer. In order to gain a greater understanding of how Akt promotes malignant transformation, we have identified likely targets for Akt. We have found that Akt phosphorylates and likely regulates the transcriptional activity of POU domain transcription factors and that Akt negatively regulates the Ras/Raf/Erk/Elk signaling pathway by phosphorylating Raf and promoting the targeted destruction of the Elk-1 transcription factor. The combination of these regulatory events are likely to be critical elements in Akt-mediated cell survival and transformation.

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15. D. H. Lee, A. L. Goldberg, *Trends Cell Biol* **8**, 397-403 (1998).
16. A. D. Kohn *et al.*, *J Biol Chem* **273**, 11937-43 (1998).

Appendix

Two publications:

1. Guan, K-L., Figueroa, C., Brtva, T., Taylor, J., Barber, T., Zhu, T. and Vojtek, A. (2000). Negative regulation of the B-Raf serine/threonine kinase by Akt. *J. Biol. Chem.* **275**, 27354-59.
2. Figueroa, C., Taylor, J., and Vojtek, A. B. (2001). PRA1 is a receptor for prenylated small GTPases. *J. Biol. Chem.*, in press.

Negative Regulation of the Serine/Threonine Kinase B-Raf by Akt*

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B-Raf contains multiple Akt consensus sites located within its amino-terminal regulatory domain. One site, Ser³⁶⁴, is conserved with c-Raf but two additional sites, Ser⁴²⁸ and Thr⁴³⁹, are unique to B-Raf. We have investigated the role of both the conserved and unique phosphorylation sites in the regulation of B-Raf activity *in vitro* and *in vivo*. We show that phosphorylation of B-Raf by Akt occurs at multiple residues within its amino-terminal regulatory domain, at both the conserved and unique phosphorylation sites. The alteration of the serine residues within the Akt consensus sites to alanines results in a progressive increase in enzymatic activity *in vitro* and *in vivo*. Furthermore, expression of Akt inhibits epidermal growth factor-induced B-Raf activity and inhibition of Akt with LY294002 up-regulates B-Raf activity, suggesting that Akt negatively regulates B-Raf *in vivo*. Our results demonstrate that B-Raf activity can be negatively regulated by Akt through phosphorylation in the amino-terminal regulatory domain of B-Raf. This cross-talk between the B-Raf and Akt serine/threonine kinases is likely to play an important role in modulating the signaling specificity of the Ras/Raf pathway and in promoting biological outcome.

Diverse extracellular stimuli, including growth factors, cytokines, and hormones, promote the formation of active, GTP-Ras. GTP-Ras directly interacts with the Raf family of serine threonine kinases and type I phosphatidylinositol 3-kinases (PI3K)¹ (1–3). Upon activation, Raf phosphorylates mitogen-activated protein kinase/extracellular signal-regulated kinase, which in turn phosphorylates and activates ERK1/2. The ERKs phosphorylate cytoplasmic targets, including the kinases Rsk and Mnk, and translocate to the nucleus where they stimulate the activity of various transcription factors, such as Elk-1, Fos, Jun, and Myc (4). Activation of phosphatidylinositol 3-kinase by both Ras-dependent and Ras-independent mechanisms leads to the increased production of phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-

trisphosphate. These lipids regulate the activity and/or localization of a number of target proteins, including those that contain pleckstrin homology domains. One such pleckstrin homology domain-containing protein regulated by lipids is the serine/threonine kinase Akt (also called protein kinase B). Akt was identified as the viral transforming agent of a T-cell lymphoma, and subsequent studies revealed a central role for Akt in promoting cell survival (5). Targets for Akt include kinases, glycogen-synthase kinase 3 and p70S6 kinase, transcription factors, FKHR and cAMP-response element-binding protein, as well as proteins associated with apoptosis, caspase 9 and BAD (6).

Ras promotes cell growth, in part by activation of the Raf/ERK pathway, and unrestrained activation of the Ras pathway is a common occurrence in many human tumors (7). In addition to its role in cell growth, Ras promotes cell survival through activation of the Raf/ERK and PI3K/Akt cascades (8). Ras also regulates differentiation; in the committed neuronal PC12 cell line, Ras/Raf promotes differentiation, whereas in C2C12 myoblasts, the Ras/Raf/ERK pathway blocks skeletal muscle differentiation (9, 10). Ras also promotes cell senescence and cell death, through activation of the Raf kinases (8). Thus, many contrary effects of Ras, promoting differentiation *versus* blocking differentiation and promoting cell proliferation, cell death, or cell survival, require the action of the Raf/ERK pathway. Cross-talk between signaling pathways is likely to be one mechanism by which such divergent biological outcomes are achieved through the use of the Ras signaling pathway. The presence of three Akt consensus sites in the amino-terminal regulatory domain of B-Raf led us to investigate cross-talk between the Ras/Raf pathway and Akt.

Mammalian cells contain three Raf proteins: c-Raf (or Raf-1), A-Raf, and B-Raf. B-Raf exists in multiple spliced forms, which exhibit tissue-specific expression patterns (11). Although all three Rafs are activated by receptor tyrosine kinases through their ability to associate with Ras, the three isoforms display differences in their regulation. Maximal activation of B-Raf requires only signals that activate Ras, whereas maximal activation of c-Raf and A-Raf require signals that activate Ras and signals that lead to their phosphorylation at tyrosine residue 341 (12, 13). Moreover, in PC12 cells, the sustained activation of ERKs in response to nerve growth factor is mediated by Rap1 acting not on c-Raf but on B-Raf (9). Thus, the three Raf proteins are differentially regulated.

Much is known about the role of phosphorylation in c-Raf regulation. Both stimulatory and inhibitory sites have been identified, and sites for serine/threonine and tyrosine phosphorylation have been mapped in c-Raf. The regulation of B-Raf by phosphorylation has diverged considerably from that of c-Raf. c-Raf contains tyrosine residues at 340 and 341, and Tyr³⁴¹ is the major site of tyrosine phosphorylation when c-Raf is coexpressed with activated Ras and Src in mammalian cells

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¹ The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HEK293 cells, human embryonic kidney 293 cells; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; K/D, kinase dead.

(13). In contrast, B-Raf does not have tyrosines at the positions equivalent to 340 and 341, but rather aspartic acid occupies these positions (Asp⁴⁴⁷ and Asp⁴⁴⁸). These aspartic acid residues in B-Raf contribute to the elevated basal kinase activity observed with B-Raf (13). The elevated kinase activity of B-Raf is normally 15–20 times higher than an equivalent amount of c-Raf. Thus, in striking contrast to c-Raf, tyrosine kinases are not involved in the activation of B-Raf. Moreover, Ser⁴⁴⁵ in B-Raf (the equivalent residue in c-Raf is Ser³³⁸) is constitutively phosphorylated (13). In c-Raf, there is a low level of phosphorylation at Ser³³⁸ in serum-starved cells, which is then greatly increased following stimulation with growth factors, including epidermal growth factor (14). Alteration of Ser⁴⁴⁵ in B-Raf reduces its basal kinase activity; thus, constitutive phosphorylation of B-Raf at Ser⁴⁴⁵, together with the aspartic acid residues at 447 and 448, is responsible for the elevated basal kinase activity of B-Raf.

Recently, while the work that is presented here was in progress, Rommel *et al.* (15) demonstrated that in one biological cell context, that of muscle cell hypertrophy, the Ras/Raf/ERK pathway and Akt have opposing effects. In addition, Zimmerman and Moelling (16) showed that Akt could phosphorylate c-Raf at Ser²⁵⁹. The alteration of Ser²⁵⁹ to A resulted in a relatively modest 2-fold increase in enzymatic activity. Phosphorylation of Ser²⁵⁹ in the amino-terminal domain of c-Raf has been previously shown to decrease the enzymatic activity of c-Raf by promoting its association with 14-3-3 proteins (12). The serine residue at 259 in c-Raf is conserved in B-Raf, Ser³⁶⁴, and as in c-Raf, this residue is part of a consensus site for Akt phosphorylation. Intriguingly, B-Raf has two additional Akt consensus sites, Ser⁴²⁸ and Thr⁴³⁹. Moreover, the two Akt consensus sites unique to B-Raf do not meet the consensus for 14-3-3 binding.

Here we demonstrate that B-Raf is phosphorylated by Akt. In contrast to c-Raf, phosphorylation of B-Raf occurs at multiple residues within its amino-terminal regulatory domain. The alteration of the serine residues within the Akt consensus sites to alanines results in a progressive increase in enzymatic activity both *in vitro* and *in vivo*. Our results demonstrate that B-Raf activity can be negatively regulated by Akt through phosphorylation in the amino-terminal regulatory domain of B-Raf.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—B-Raf was expressed as a fusion protein in HEK293 cells to either glutathione S-transferase (GST) in the expression vector pEBG-4X or to HA in the expression vector pcDNA3 (Invitrogen). Mutations in B-Raf at the ATP binding site (K482M) and at the Akt consensus phosphorylation sites were generated by site-directed mutagenesis and confirmed by restriction enzyme and sequence analysis.

Kinase Assays—For HA-B-Raf activity assays, 100 ng of pcDNA3-HA-B-Raf was transfected into HEK293 cells in 6-well plates using LipofectAMINE (Life Technologies, Inc.). 24 h after transfection, cells were starved in 0.1% fetal bovine serum medium for 12 h. Cells were lysed in radioimmune precipitation buffer. HA-B-Raf was immunoprecipitated with 2 μ g of anti-HA antibody (BabCo). The immunoprecipitated HA-B-Raf was assayed by a coupled *in vitro* kinase assay (17). Briefly, 0.1 μ g of GST-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 was incubated with the precipitated HA-B-Raf in 20 μ l of kinase buffer for 20 min at 30 °C. The reaction was briefly centrifuged to pellet the HA-B-Raf, which was bound to protein G-agarose. 15 μ l of the supernatant was mixed with 0.1 μ g of GST-ERK1 in 20 μ l of kinase reaction buffer and incubated for 10 min at 30 °C. Then 10 μ l of a mixture containing 3 μ g of GST-Elk-1 and [γ -³²P]ATP was added to the reaction and incubated for 20 min at 30 °C. Phosphorylation of GST-Elk was determined by SDS-polyacrylamide gel electrophoresis and phosphorimage analysis.

Myc-ERK plasmid (300 ng) was transfected into HEK293 cells in the presence or absence of B-Raf or Ras. 24 h after transfection, cells were starved in 0.1% fetal bovine serum medium for 12 h. Myc-ERK were

immunoprecipitated, and kinase activity was determined using GST-Elk as a substrate (18). The amount of Myc-ERK used for kinase assays was analyzed by anti-ERK immunoblot.

For the Akt kinase assays, HEK293 cells in 60 mm dishes were transfected with 6 μ g of pCS2+-N-FLAG-Akt using a calcium phosphate transfection method. 48 h after transfection, cells were starved for 16 h in 0.1% fetal bovine serum medium. Following treatment with 100 mM LY294002 (or Me₂SO vehicle) for 1 h, cells were stimulated with insulin (20 μ g/ml medium; 3.5 μ M) for 10 min and immediately lysed in buffer A (19). Clarified extracts were incubated for 2 h with anti-FLAG M2-agarose resin to purify FLAG-Akt. The immunoprecipitated Akt was washed three times in buffer A containing 0.5 M NaCl, twice with buffer B, and once with assay dilution buffer before being evenly aliquotted to tubes for kinase assay. GST-tagged, kinase-inactive B-Raf and B-Raf mutant cDNAs (15 μ g) were transfected into 100-mm dishes of HEK293 cells. 36 h after transfection, actively growing cells were lysed in radioimmune precipitation buffer, and GST-B-Raf proteins were collected with glutathione-Sepharose beads. The beads were washed three times in buffer A containing 0.5 M NaCl, twice with buffer B, and GST-B-Raf substrates were then eluted using 10 mM glutathione in 50 mM Tris (pH 8.0) and quantitated using a Bradford assay (Bio-Rad). Approximately 1.0 μ g of each GST-B-Raf mutant was incubated with immunoprecipitated Akt at 30 °C for 30 min in a kinase reaction containing 6.7 mM MOPS (pH 7.2), 8.3 mM β -glycerol phosphate (pH 7.0), 0.33 mM Na₂VO₄, 0.33 mM dithiothreitol, 25 mM MgCl₂, 167 mM ATP, and 10 μ Ci of [γ -³²P]ATP. One-third of the reaction product was subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to Immobilon filter, phosphorimager analysis, and Western blotting to verify equivalent GST-B-Raf substrate levels.

Coimmunoprecipitation of B-Raf and Akt—GST-Akt and HA-B-Raf were cotransfected into HEK293 cells using a calcium phosphate transfection protocol. Approximately 36 h after transfection, cells were lysed in 10 mM HEPES, pH 7.4, 50 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% β -mercaptoethanol, 1% aprotinin, 50 mM NaF. Glutathione-agarose beads were added to the cell lysates to purify GST-Akt and associated protein(s). GST-Akt and associated protein(s) were eluted in 10 mM glutathione in 50 mM Tris, pH 8.0. Similar experiments were performed with cells transfected with GST-B-Raf and HA-Akt, except that the cells were lysed in 10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mg/ml leupeptin, 5 mg/ml aprotinin, 50 mM NaF, 1 mM sodium vanadate. A GST expression vector was included as a negative control. The glutathione-eluted samples were analyzed by Western blot with anti-GST and anti-HA antibodies.

Reporter Assays—In general, HEK293 cells in 35-mm dishes were transfected using a standard calcium phosphate transfection protocol with 36 ng of Gal4-ElkC chimera, 290 ng of a 5xGal4-luciferase reporter, and 145 ng of a B-Raf expression vector or 15 ng of a K-RasV12 expression vector. Total DNA was kept constant by the addition of the appropriate amount of pcDNA3 for all transfections. Luciferase assays were performed using the dual-light luciferase and β -galactosidase reporter gene assay system (Tropix) and were normalized for transfection efficiency using a cotransfected β -galactosidase expression vector (15 ng).

RESULTS

The presence of multiple Akt consensus sites in the amino-terminal regulatory domain of B-Raf led us to investigate whether cross-talk between the Ras signaling pathway and Akt occurs *in vivo*. To determine whether Akt can modulate B-Raf function *in vivo*, we examined the effect of Akt activation and inhibition on B-Raf activity (Fig. 1). Expression of a membrane localized, constitutively active Akt (myrAkt) inhibits B-Raf activity in both actively growing HEK293 cells (Fig. 1A, compare lane 2 with lane 1) and in cells stimulated with epidermal growth factor (Fig. 1A, compare lane 3 with lane 4). The inhibition of B-Raf activity by Akt is dose-dependent (Fig. 1B). A kinase dead version of Akt has no effect on B-Raf activity, suggesting that the catalytic activity of Akt is required to inhibit B-Raf (Fig. 1C). To further test the involvement of endogenous Akt in B-Raf regulation, the PI3K inhibitor LY294002 was used to block the PI3K/Akt pathway. As shown in Fig. 1D, the addition of LY294002 elevates B-Raf activity, suggesting that Akt negatively regulates B-Raf enzymatic activity.

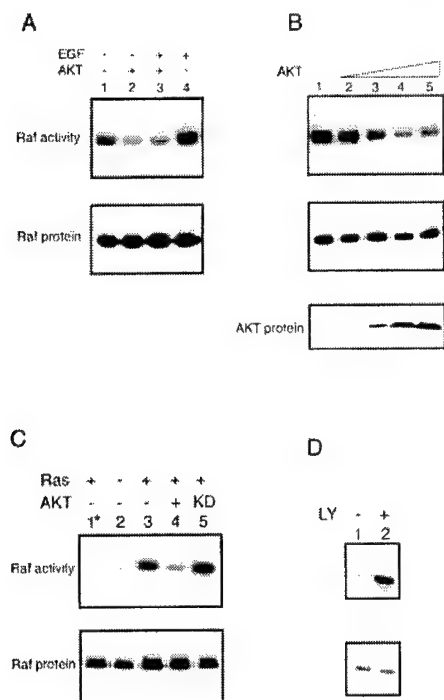


FIG. 1. Akt inhibits B-Raf activity *in vivo*. A, HEK293 cells were transfected with expression vectors for HA-B-Raf and myrAkt, as indicated. B-Raf was immunoprecipitated from lysates and Raf activity assessed by an *in vitro*-coupled kinase reaction in which bacterially expressed GST-Elk-1 was used as a substrate (top panel). Lanes 1 and 2, B-Raf was immunoprecipitated from transfected cells. Lanes 3 and 4, B-Raf was immunoprecipitated from cells after serum starvation and stimulation with epidermal growth factor for 3 min. Western blot of HA-B-Raf proteins used for the *in vitro* kinase is shown in the bottom panel. B, Akt inhibits Raf enzymatic activity in a dose-dependent manner. HEK293 cells were transfected with the expression vector for HA-B-Raf and increasing concentrations of myrAkt, as indicated. B-Raf activity was determined by a coupled *in vitro* kinase reaction (top panel). Western blot of HA-B-Raf proteins used in the *in vitro* kinase reactions (middle panel). Western blot of HA-Akt in cell lysates (bottom panel). C, Akt kinase activity is required to inhibit B-Raf activity. HA-B-Raf was transfected with expression vectors for K-RasV12 or Akt as indicated. Activated Ras stimulates the co-transfected Raf activity (compare lanes 2 and 3). GST-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase was omitted in lane 1 as a control for the specificity of the *in vitro* Raf kinase assays. The Ras-induced Raf activation is inhibited by wild type (lane 4) but not the kinase inactive (lane 5) Akt. D, B-Raf activity is enhanced upon inhibition of PI3K. HA-B-Raf-transfected cells were treated with LY294002 for 1 h. HA-B-Raf was isolated, and kinase activity was determined.

We also examined the association of B-Raf with Akt *in vivo*. As shown in Fig. 2, B-Raf and Akt associate in HEK293 cells overexpressing these proteins. The association between Akt and B-Raf is observed in actively growing HEK293 cells and in HEK293 cells that have been serum-starved or serum-starved and then stimulated with insulin. However, in one orientation, when GST-B-Raf is isolated from HEK293 cells expressing Flag epitope-tagged Akt, the association between B-Raf and Akt is enhanced upon insulin stimulation (data not shown). This may suggest that the association between B-Raf and Akt is sensitive to, but not absolutely dependent on, whether one or both of the proteins is in an activated state.

B-Raf contains three Akt consensus sites, Table I. One site, Ser³⁶⁴ is conserved with c-Raf; however, two sites, Ser⁴²⁸ and Thr⁴³⁹, are unique to B-Raf. To begin to examine the physiological significance of phosphorylation at these sites, the conserved and unique sites were altered to alanine alone and in

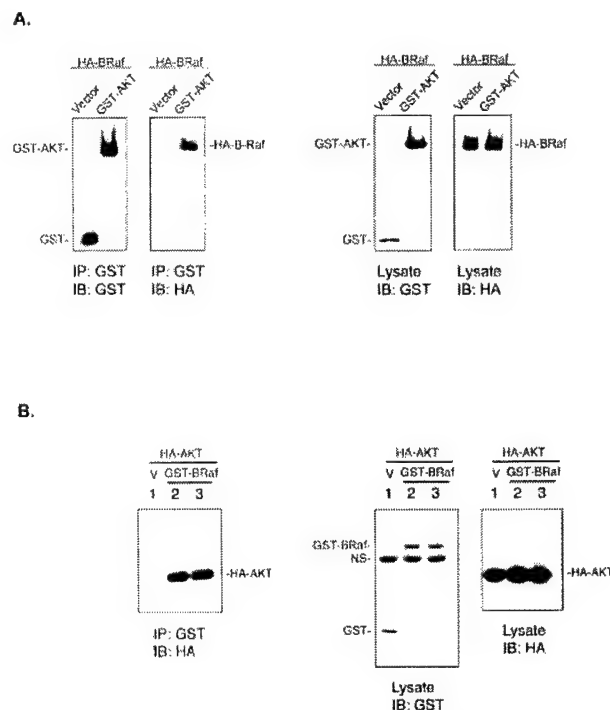


FIG. 2. B-Raf and Akt co-associate *in vivo*. GST-Akt (A) or GST-B-Raf (B) was purified from lysates prepared from HEK293 cells transfected with the indicated constructs using glutathione-Sepharose. The pull-downs were subject to SDS-polyacrylamide gel electrophoresis followed by Western blotting with antibodies directed against the epitope tags on B-Raf and Akt. IP, immunoprecipitate; IB, immunoblot.

TABLE I
Akt consensus sites RXXRXXS/T ϕ

Protein	Site	Position
Glycogen-synthase kinase 3 β	RPRTTSF	9
BAD	RGRSRSA	136
FKHR	RRRAASM	253
c-Raf	RQRSTST	259
B-Raf	RDRSSSA	364
	RERKSSS	428
	RNRMKTL	439

combination. The effect of the mutations on enzymatic activity *in vitro* and *in vivo* was assessed.

Mutation of Ser³⁶⁴ to Ala (A), of both 428 and 439 to Ala (AA), or of all three Akt consensus sites to Ala (AAA) resulted in a progressive increase in enzymatic activity: B-Raf < B-Raf A < B-Raf AA < B-Raf AAA (Fig. 3). Alteration of Ser³⁶⁴ to Ala (conserved with c-Raf) leads to a modest 2X elevation in enzymatic activity, whereas alteration of both Ser⁴²⁸ and Thr⁴³⁹ to Ala (unique to B-Raf) leads to at least a 19 times elevation of enzymatic activity. Thus, phosphorylation of B-Raf at both the unique and conserved phosphorylation sites is likely to negatively regulate B-Raf enzymatic activity.

Activation of the Ras/Raf/ERK pathway culminates in the phosphorylation of transcription factors, including the ternary complex factor Elk-1 (4). Thus, the degree of activation of the Ras/Raf/ERK pathway can be assessed by examining the transcriptional activity of Elk-1. To address whether the increase in enzymatic activity of the B-Raf alanine mutants *in vitro* correlates with deregulation of enzymatic activity *in vivo*, the effect of the B-Raf mutants on Elk-1-mediated transcriptional activity was assessed by examining the activity of a Gal4-ElkC reporter. Gal4-ElkC contains the carboxyl-terminal domain of

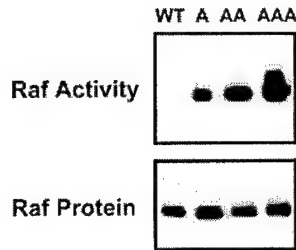


FIG. 3. B-Raf kinase activity is progressively increased upon mutation of the Akt consensus sites. HA epitope-tagged B-Raf, wild type, or mutant at the Akt consensus sites was immunoprecipitated from HEK293 cells, and its enzymatic activity assessed by a coupled *in vitro* kinase reaction in which bacterially expressed GST-Elk-1 was used as substrate. WT, wild type HA-B-Raf; A, HA-B-Raf S364A; AA, HA-B-Raf S428A/T439A; AAA, S364A/S428A/T439A. Raf kinase activity and protein are shown in the *top* and *bottom* panels, respectively.

Elk-1, which includes the transactivation domain of Elk and the ERK phosphorylation sites, fused to the DNA binding domain of the yeast Gal4 protein. The transcriptional activity of Gal4-ElkC is dependent on the phosphorylation of the Elk transactivation domain by ERK (20). As shown in Fig. 4, the B-Raf mutants activate the Elk reporter to a greater extent than wild type B-Raf. Furthermore, B-Raf mutant at all three of the Akt consensus sites activates the Elk-1 reporter to a greater extent than B-Raf mutant at only 364 or B-Raf mutant at both S428A and T439A. The observed increase in reporter activity with the mutants correlates with the progressive increase in enzymatic activity observed in the *in vitro* kinase reactions shown in Fig. 3. Thus, B-Raf proteins altered so as to prevent phosphorylation at the Akt consensus sites activate downstream signaling events.

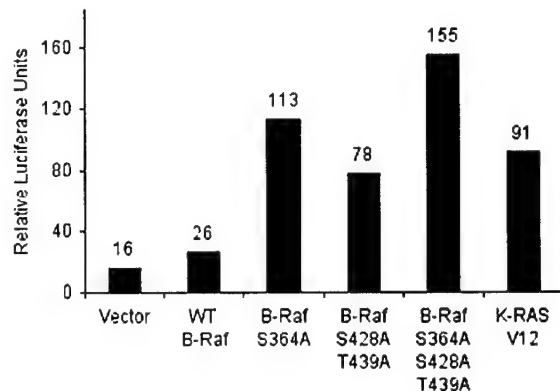
We also examined the effect of the phosphatidylinositol 3-kinase inhibitor LY294002 on activation of the Elk-1 reporter by wild type B-Raf. We consistently observe a modest increase in the ability of B-Raf to activate the Elk-1 reporter in the presence of LY294002 (Fig. 4). This result is consistent with the data in Fig. 1 showing that Akt can negatively regulate B-Raf activity *in vivo*.

In addition, we assessed the deregulation of the enzymatic activity of the B-Raf mutants *in vivo* by assaying ERK activity in the presence and absence of the B-Raf mutants. As expected, ERK activity is elevated in HEK293 cells expressing the B-Raf mutants: B-Raf AAA > B-Raf AA > B-Raf A > B-Raf (Fig. 5).

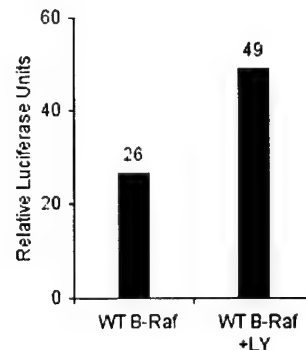
To determine whether B-Raf is a substrate of Akt, *in vitro* kinase reactions were performed. A kinase dead version of B-Raf fused to glutathione *S*-transferase (K/D GST-B-Raf) was used in the *in vitro* kinase reactions. Akt phosphorylates K/D GST-B-Raf but not the control protein GST (Fig. 6). Phosphorylation of K/D GST-B-Raf requires active Akt because phosphorylation was not observed when Akt was prepared from cells in the presence of the phosphatidylinositol 3-kinase inhibitor LY294002.

To determine which of the Akt consensus sites in B-Raf were utilized by Akt, each of the sites was altered to alanine alone and in combination in the context of a K/D GST-B-Raf (see Table I). Each of the mutant proteins was purified from HEK293 cells and used as substrate in *in vitro* kinase reactions with active Akt (harvested from cells after insulin stimulation) or inactive Akt (harvested from cells after treatment with LY294002 and insulin) (Fig. 6A). Quantitation of the *in vitro* kinase reactions by phosphorimage analysis indicates that each of the single mutants B-Raf S364A and B-Raf S428A is phosphorylated to approximately the same extent and that the extent of phosphorylation of each of these mutants is approxi-

A.



B.



C.

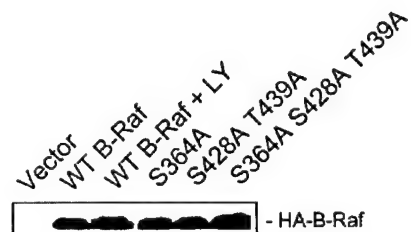


FIG. 4. Activation of Elk-mediated transcription by B-Raf mutants. A, HEK293 cells were transfected with expression vectors for Gal4-ElkC, Gal4-luciferase, and either pcDNA3 (vector control), K-Ras V12 (positive control), wild type (WT)-B-Raf, B-Raf S364A, B-Raf S428A/T439A, or B-Raf S364A/S428A/T439A. B, LY294002 (LY) was added for 24 h prior to harvest, WT B-Raf + LY. Luciferase activity was assayed 36 h after transfection. Luciferase activity was normalized to a co-transfected β -galactosidase expression vector. Shown is the average of two experiments performed in duplicate. C, Western blot of extracts showing expression of HA-B-Raf wild type and mutants.

mately one half that observed for the B-Raf control (Fig. 6B). Taken together, these observations suggest that Akt phosphorylates both Ser³⁶⁴ and Ser⁴²⁸. In contrast, the single mutant B-Raf T439A is not significantly decreased in its phosphorylation compared with the B-Raf control, suggesting that Thr⁴³⁹ is not subject to phosphorylation by Akt. Consistent with the idea that Thr⁴³⁹ is not phosphorylated by Akt, the double B-Raf mutant S428A/T439A is phosphorylated to the same extent as the single B-Raf mutant S428A. Moreover, the extent of phosphorylation of the triple B-Raf mutant S364A/S428A/T439A is approximately one half that observed for each of the single mutants at residues 364 or 428, confirming that Akt phospho-

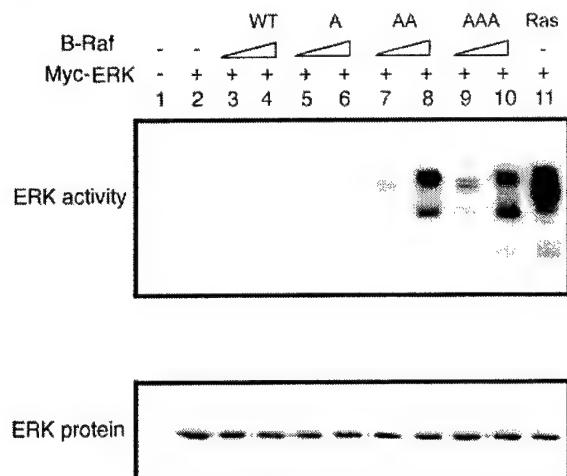


Fig. 5. Mutant Raf has enhanced ability to stimulate ERK activity *in vivo*. HEK293 cells were transfected with expression vectors for wild type or mutant B-Raf proteins or RasV12 and Myc-ERK, as indicated. Myc-ERK was immunoprecipitated, and kinase activity was determined. Western blotting with anti-ERK antibody shows the level of Myc-ERK used in the kinase assay (bottom panel).

rylates both Ser³⁶⁴ and Ser⁴²⁸. Thus, Akt phosphorylates B-Raf *in vitro* at residues Ser³⁶⁴ and Ser⁴²⁸.

DISCUSSION

Here we have demonstrated that *in vivo* and *in vitro* the Ras/Raf signaling pathway is negatively regulated by Akt. First, epidermal growth factor stimulation of B-Raf activity is inhibited by co-expression of Akt. Second, B-Raf enzymatic activity is elevated after treatment of cells with LY294002, a pharmacological inhibitor of PI3K/Akt. Taken together, these observations demonstrate that Akt down-regulates B-Raf activity *in vivo*. Because B-Raf contains three Akt consensus sites located within its amino-terminal regulatory domain, the most likely effect of Akt is a direct phosphorylation at one or more of these sites and a concomitant down-regulation of B-Raf enzymatic activity. We have demonstrated that Akt will phosphorylate two of the three Akt consensus phosphorylation sites *in vitro*, Ser³⁶⁴ and Ser⁴²⁸. Ser³⁶⁴ is conserved with c-Raf, but Ser⁴²⁸ is unique to B-Raf. Substitution of the phosphorylatable residue in the Akt consensus phosphorylation sites with alanine increased B-Raf enzymatic activity, as assessed by *in vitro* coupled kinase assays, activation of the Elk-1 reporter, and activation of ERK activity. Thus, phosphorylation of B-Raf at multiple residues within the amino-terminal regulatory domain negatively regulates its enzymatic activity *in vitro* and *in vivo*.

B-Raf enzymatic activity is further enhanced by combining the alanine substitution mutations within the Akt consensus phosphorylation sites. The seemingly additive nature of the mutations suggests that phosphorylation of these residues is not likely to be ordered but rather phosphorylation at one residue is likely to occur independently of the status of phosphorylation at the other residues. The multiplicity of phosphorylation sites in B-Raf may enable a more flexible regulation (either duration or timing) of B-Raf activity.

Surprisingly, blocking the activation of Akt with the pharmacological inhibitor of phosphatidylinositol 3-kinase, LY294002, did not up-regulate B-Raf activity to the same extent as altering all three Akt consensus sites. The addition of LY294002 only modestly up-regulated B-Raf enzymatic activity (Fig. 1 and data not shown) and Elk-1 reporter activity (Fig. 4), whereas the B-Raf AAA mutant exhibited a striking 20× elevation in enzymatic activity (Fig. 3) and a 6× elevation in

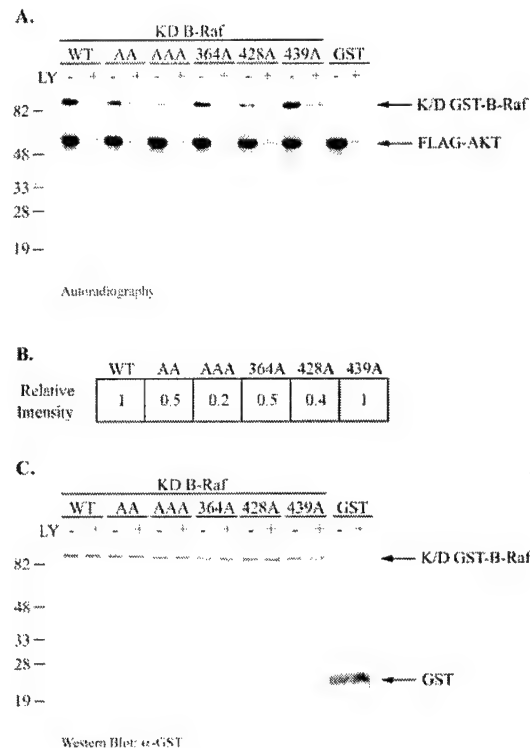


Fig. 6. B-Raf is phosphorylated *in vitro* by Akt at residues 364 and 428. Kinase inactive (K/D) GST-B-Raf fusion proteins were subjected to an *in vitro* kinase reaction with active Akt (-LY) or inactive Akt (+LY). In addition to the K482M mutation to create a kinase-inactive B-Raf, the B-Raf proteins either do not contain additional mutations and are designated WT, for wild type at the Akt consensus sites, or contain mutations at the Akt consensus sites alone (S364A, S428A, or T439A) or in combination (AA, S428A/T439A; AAA, S364A/S428A/T439A). A, autoradiography of the *in vitro* kinase reactions after SDS-polyacrylamide gel electrophoresis and transfer to an Immobilon filter. Arrows show the position of the phosphorylated K/D GST-B-Raf and the autophosphorylated Flag-Akt. B, relative intensity of the phosphorimage signal (-LY lanes) of wild type and mutant K/D GST-B-Raf from the filter in A. The intensity of the signal for each of the mutant proteins was determined relative to K/D GST-B-Raf, which was set to 1.0. C, Western blot analysis of the filter in A, using an anti-GST antibody showing equal loading of the GST-B-Raf substrates.

Elk-1 reporter activity (Fig. 4). Possibly the addition of LY294002 does not block Akt activity in its entirety, a result consistent with observations that Akt can be activated by lipid-independent mechanisms (6). Alternatively, other kinases, in addition to Akt, may be able to regulate B-Raf enzymatic activity by phosphorylation of these sites. Because Thr⁴³⁹ is not phosphorylated by Akt *in vitro*, these other kinases may negatively regulate B-Raf activity at this site.

While our studies were in progress, c-Raf was shown to be phosphorylated by Akt at Ser²⁵⁹ (16), equivalent to Ser³⁶⁴ in B-Raf. The alteration of Ser²⁵⁹ to alanine in c-Raf resulted in a modest 2-fold increase in enzymatic activity. Phosphorylation of Ser²⁵⁹ in c-Raf had been previously shown to decrease the enzymatic activity of c-Raf by promoting its association with 14-3-3 proteins (12). Our results support and extend these observations. First, the regulation of Raf kinases extends to multiple family members; both c-Raf (16) and B-Raf (this report) are subject to phosphorylation and regulation by Akt. Second, of the three Akt consensus sites in B-Raf, only the site conserved with c-Raf (Ser³⁶⁴) lies within a 14-3-3 binding motif (RSXS*XP, where S* represents phosphorylated serine) (12). This suggests that an as yet uncharacterized molecular mechanism, in addition to 14-3-3 binding, is contributing to the

negative regulation of B-Raf at Ser⁴²⁸. Phosphorylation of B-Raf by Akt does not appear to disrupt the association between B-Raf and mitogen-activated protein kinase/extracellular signal-regulated kinase or heat shock protein 90.² Phosphorylation may decrease the association between B-Raf and Ras or Rap or other adaptors and/or signaling molecules, such as Raf kinase inhibitor protein, that regulate Raf activity. Alternatively, phosphorylation may hinder the association of B-Raf with the plasma membrane. Experiments are in progress to test these possibilities. Third, the discordance between the activity of the B-Raf protein mutant at the Akt consensus sites and the activity of B-Raf in the presence of LY294002 might suggest that kinases, in addition to Akt, can act to negatively regulate B-Raf at one or more of these residues.

The Ras signaling pathway regulates cell growth, differentiation, cell survival, cell senescence, and cell death. The sustained *versus* transient activation of the Ras/Raf/ERK pathway is a critically important mediator of signaling specificity and biological outcome (9, 21). We have demonstrated here that Akt can regulate the activity of the kinase cascade downstream of Ras through phosphorylating and inhibiting B-Raf activity. It seems likely that the integration of signals from multiple kinase cascades within a particular cell, such as described here for B-Raf and Akt, will play an important role in modulating the specificity and biological outcome of signal transduction pathways.

² K.-L. Guan, C. Figueroa, and A. B. Vojtek, unpublished observations.

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PRA1 is a receptor for prenylated small GTPases

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Running title: PRA1 associates with multiple Ras family proteins

Summary

Localization of Ras and Ras-like proteins to the correct subcellular compartment is essential for these proteins to mediate their biological effects. Many members of the Ras superfamily (H-Ras, N-Ras, TC21, RhoA) are prenylated in the cytoplasm and then transit through the endomembrane system on their way to the plasma membrane. The proteins that aid in the trafficking of the small GTPases have not been well characterized. We report here that PRA1, which others previously identified as a prenylation-dependent receptor for Rab proteins, also interacts with Ha-Ras, RhoA, TC21 and Rap1a. The interaction of these small GTPases with PRA1 requires their post-translational modification by prenylation. The prenylation dependent association of PRA1 with multiple GTPases is conserved in evolution; the yeast PRA1 protein associates with both Ha-Ras and RhoA. Earlier studies reported the presence of PRA1 in the Golgi and we show here that PRA1 co-localizes with Ha-Ras and RhoA in the Golgi compartment. We suggest that PRA1 acts as an escort protein for small GTPases by binding to the hydrophobic isoprenoid moieties of the small GTPases and facilitates their trafficking through the endomembrane system.

Introduction

Ras proteins (H-Ras, K-Ras, N-Ras, TC21, Ras1/2) regulate cell growth in eukaryotic cells and perturbation of signaling pathways by mutation and constitutive activation of Ras proteins is a common occurrence in a wide spectrum of human tumors (1). In addition to regulating cell proliferation, Ras proteins also regulate differentiation, cell death and cell survival (1). The Ras proteins are members of a large superfamily of low molecular weight GTP binding proteins, which include members that regulate the actin cytoskeleton (Rho, Rac), vesicle trafficking (Rabs), and nuclear transport (Ran).

The biological activity of Ras proteins is controlled by a regulated GTP/GDP cycle (2). The GTP-bound, or active Ras, relays signals to downstream effector proteins. In the case of Ha-Ras, numerous effector proteins have been described, including serine/threonine kinases (c-Raf, A-Raf, B-Raf), lipid kinases (type I phosphatidylinositol 3-kinase), and guanine nucleotide dissociation stimulators for Ral (Ral GDS¹, RGL) (1). Activation of Ras effector pathways leads to proliferation, differentiation, cell death and cell survival. Which biological outcome predominates is somewhat of a mystery but seems to depend on cell type and the coordinate integration of signaling pathways activated and/or inhibited within a cell.

Members of the Ras superfamily are subject to post-translational modifications. The spectrum of modifications depends on the composition of the carboxyl terminus. In the case of Ha-Ras, the protein is subject to prenylation, proteolysis, carboxylmethylation, and S-acylation/palmitoylation (3). Prenylation is the covalent attachment of farnesyl or

¹ The abbreviations used are: ER, endoplasmic reticulum; GDI1, guanine nucleotide dissociation inhibitor 1; GDS, guanine nucleotide dissociation stimulator; GST, glutathione S-transferase; PRA1, prenylated Rab acceptor protein; and, SOS, son of sevenless.

geranylgeranyl isoprenoids at or near the carboxyl terminus of the GTPase. For Ras family members, prenylation occurs at a conserved cysteine in the carboxy terminal motif termed the CAAX box, where C is cysteine, A is an aliphatic amino acid, and X is usually S, M, Q or L(3) (4). Whereas Ha-Ras, N-Ras, K-Ras4B and yeast Ras2 are farnesylated, most other small GTPases are geranylgeranylated (5). RhoB can be either farnesylated or geranylgeranylated (5). The enzymes that catalyze prenylation, farnesyltransferase and geranylgeranyltransferase-I and II, reside in the cytoplasm.

Prenylation is followed by proteolysis of the carboxy terminal tripeptide and methylation of the newly generated carboxy terminal amino acid in the endoplasmic reticulum (ER) (3) (4). Some Ras proteins, including Ha-Ras, N-Ras, yeast Ras2 and Rac1 are further modified by S-acylation (palmitoylation) of cysteine(s) located near the carboxy terminus of the protein (3) (4). S-acylation is likely to occur in the ER since Erf2, a novel integral membrane protein required for palmitoylation of Ras in yeast, is localized to the ER (6). Other proteins, such as K-Ras4B, which are not subject to S-acylation, have a polybasic stretch of amino acids, also located near the CAAX box, within the 20 amino acid hypervariable domain of the Ras proteins. The combination of two signals, prenylation and S-acylation or prenylation and a polybasic stretch of amino acids, are required for plasma membrane targeting of Ras proteins. Ras proteins (Ha-Ras, N-Ras) transit through the ER and Golgi and finally reach the plasma membrane via exocytic transport vesicles (7) (8). The trafficking of K-Ras4B, however, is unlikely to involve the Golgi compartment; rather, once prenylated and further modified in the ER, the trafficking of K-Ras4B to the plasma membrane involves a microtubule network (9) (10).

As a consequence of the post-translational modifications, Ras is membrane localized. In mammalian cells, membrane localization of Ras is necessary for transformation and differentiation. Membrane localization of Ras creates a docking site for effectors, including the Raf kinases and phosphatidylinositol 3-kinase. Membrane localization of Raf may also facilitate and/or enable the subsequent events needed for activation of its catalytic activity, including interactions with phospholipids and phosphorylation by serine/threonine and tyrosine kinases. Membrane localization of phosphatidylinositol 3-kinase may accelerate its enzymatic activity by localizing the enzyme to a site rich in substrate lipids. In *Saccharomyces cerevisiae*, membrane localization of Ras is required for the transient increase in cAMP in response to glucose addition, but is not required for Ras to fulfill its essential function in the cell (11).

A number of studies have demonstrated that prenylation of Ras is a critical determinant in initiating membrane attachment. Without prenylation, the subsequent processing events do not take place and the Ras proteins remain soluble (4) (3). In addition to initiating the process that ultimately leads to membrane attachment of the small GTPases, farnesylation may also contribute to protein-protein interactions. Studies using an activated Ras2 protein mutated at the farnesylation site reported a decreased interaction between the mutant Ras2 and its effector in yeast, adenylyl cyclase, suggesting that the farnesyl moiety itself or the conformation imposed on the protein by modification may play a role in mediating productive Ras-effector interactions (12). In addition, in vitro studies have suggested that farnesylation of Ras may be needed for SOS to promote nucleotide exchange (13). Finally, although farnesylation by itself is not sufficient to target proteins to the plasma membrane, this modification may contribute to stable membrane binding (23). Taken together, these observations suggest that prenylation is crucial for Ras function.

Using a yeast two hybrid screen, we identified Ha-Ras interacting proteins (RIPs) (14). We demonstrate in this report that one of these proteins, Rip69, interacts with multiple members of the Ras superfamily, including Ha-Ras, RhoA, and TC21, and that the interaction of Rip69 with the small GTPases requires their post-translational modification by prenylation. Rip 69 encodes residues 20 to 186 of PRA1, prenylated Rab acceptor protein. PRA1 was identified as a Rab interacting protein in yeast two hybrid screens and PRA1 was reported to interact with Rab proteins but not other members of the Ras superfamily (15) (16) (17). In addition to binding Rab proteins, PRA1 also associates with GDP dissociation inhibitor 1 (GDI1) and the v-SNARE VAMP2 (16) (15). In vitro PRA1 can block the ability of GDI1 to extract Rab3A from membranes and, therefore, the opposing actions of GDI1 and PRA1 on Rab proteins may influence the membrane localization of the Rab proteins (16). Further, Rab3A can displace VAMP2 from VAMP2-PRA1 complexes, so displacement of PRA1 from VAMP2 by Rab may also facilitate v-SNARE/t-SNARE interactions and vesicle fusion (15).

With this report, we demonstrate that PRA1 is not a specific partner for Rab proteins but also partners with other members of the Ras superfamily. The association of Ras family members with PRA1 requires their post-translational modification by prenylation. Further, our results suggest that an isoprenoid moiety, either farnesyl or geranylgeranyl, is the critical recognition target for PRA1. The interaction between Ha-Ras and PRA1 and between RhoA and PRA1 is observed in vivo by co-precipitation experiments and co-localization in mammalian cells. The co-localization of Ha-Ras and PRA1 and RhoA and PRA1 to the Golgi compartment suggests that PRA1 may play a role in facilitating the trafficking of small GTPases through the endomembrane system. In addition, we have cloned the yeast PRA1 gene and demonstrate that, like its higher eukaryotic counterpart, the yeast PRA1 protein also interacts with multiple small

GTPases and the interaction requires prenylation of the small GTPase. Thus, PRA1 is predicted to play a conserved role in the biology of small GTPases in all eukaryotic cells.

Experimental Procedures

Plasmids. Rip69 was identified in a yeast two hybrid screen of a 9.5 and 10.5 day short insert size, random primed mouse embryo library using LexA-Ha-RasV12 as bait (14). Rip69 encodes residues 20 to 186 of PRA1 fused in frame to a nuclear localized VP16 protein in pVP16.

pLexA-Ha-Ras V12, pLexA-RhoA L63, pLexA-TC21, and pLexA-Rap1a have been described (14) (18); these plasmids all express the full length small GTPase fused in frame to the LexA DNA binding domain in pLexA or pLexA-Ade. pLexA-Ha-RasV12K6 and pLexA-Ha-RasV12 S181 S184 were constructed by inserting a BamHI-EcoRI fragment generated by polymerase chain reaction (PCR) using a Ha-RasV12 template, Expand polymerase (BM), and the following forward and two reverse primers, respectively: 5'CGGAATTCATGACGGAATATAAGCTGG 3' and 5'CGGGATCCTCACTTCTTCTTCTTCTTCTTGAGCACACACTTGCAGCT3' or 5'CGGGATCCTCAGGAGAGCACACACTTGGAGCTCATGGAGCCGGGGCCAC3.'

pLexA-GFP and pLexA-GFPCAAX were generated by PCR using pCS2+eGFP BglII as template and the following forward and two reverse oligonucleotide primers:

5'CGGGATCCGCACCATGGTGAGCAAGGGCGAG3' and

5'CGGAATTCTTGCGGCCGCAATTATCCACCGCCCTTGTACAG3' or

5'CGGAATTCTTGCGGCCGCAATTAGGAGAGCACACATCCACCGCCCTTGTACAG3.'

Inserts generated after PCR were sequenced after subcloning. Ha-RasV12 and RhoA L63 were expressed as fusion proteins in HEK293 cells to glutathione S-transferase (GST) in the expression vector pEBG-3X. Full length yeast PRA1 was isolated from yeast genomic DNA

using PCR with gene specific oligonucleotide primers with appropriate restriction sites for subcloning into pVP16. Full length mouse PRA1 was isolated by reverse transcription-polymerase chain reaction (RT-PCR) from mouse brain RNA, as described (19), using gene specific oligonucleotide primers. The full length mouse PRA1 cDNA was subcloned into pCS2+MT and sequenced.

Yeast two-hybrid assays. The *Saccharomyces cerevisiae* strain L40 was transformed with plasmids expressing fusions to the LexA DNA binding domain in pLexAde or pLexAdeNot and with plasmids expressing fusions to a nuclear localized VP16 acidic activation domain in pVP16 (14). Transactivation of the *His3* reporter was assessed by growing yeast overnight while maintaining selection for the plasmids and then plating ten fold serial dilutions to YC-WHULK and YC-LW plates. The plates were incubated for 3 days at 30°C and then photographed.

Co-precipitation of PRA1 with Ha-Ras, RhoA or Rap1a. GST-Ha-RasV12, GST-RhoAL63, or GST-Rap1a and MT-PRA1 were co-transfected into HEK293 cells using a calcium phosphate transfection protocol. Approximately 36 hours after transfection, cells were lysed in Triton IP buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 1% triton X-100, 2 mM EDTA, 0.1% β -mercaptoethanol, 1% aprotinin, 50 mM NaF, 1 mM PMSF). Glutathione agarose beads were added to the cell lysates to purify GST-Ha-Ras, GST-RhoA, or GST-Rap1a and associated protein(s). The beads were washed three times with Triton IP buffer and suspended in sample buffer. A GST expression vector was included as a negative control. The proteins bound to glutathione sepharose beads were analyzed by western blotting with anti-GST and anti-myc tag epitope antibodies.

In vitro protein-protein interactions. HEK293 cells were transfected with expression vectors for GST-Ha-RasV12 and GST, pEBG3X-Ha-RasV12 or pEBG, respectively. Processed and unprocessed GST-Ha-Ras proteins were prepared by Triton X-114 partitioning essentially as described (20) followed by purification of the processed and unprocessed GST-Ras proteins on glutathione sepharose resin. VP16-Rip69 and VP16 proteins were prepared in the presence of 35-S methionine by coupled in vitro transcription-translation (TNT, Promega) of the appropriate expression vector, pRip69 or pVP16. The labeled Rip69 and VP16 proteins were incubated with glutathione sepharose bound processed GST-Ha-Ras protein, unprocessed GST-Ha-Ras protein, or control GST protein. After incubation for 1 hour at 4°C, the resin was washed three times in Triton IP buffer. The GST-fusion proteins bound to glutathione sepharose beads and any co-associated proteins were subjected to SDS-PAGE and transferred to Immobilon. 35-S methionine labeled Rip69 was detected by autoradiography. The GST-fusion proteins were detected by western blot analysis using an anti-GST antibody.

Microscopy. Cos cells were transiently transfected with expression vectors for eGFP-PRA1 and either GST-Ha-Ras or GST-RhoA, pCS2+eGFP-PRA1, pEBG-3X-Ha-RasV12 and pEBG-RhoAL63, respectively. Cells were fixed 36 hours post-transfection on polylysine coated coverslips with 3.7% paraformaldehyde (Sigma) in phosphate buffered saline (PBS), permeabilized for 15 minutes with 0.1% Triton X-100 in PBS, and blocked with 10% goat serum in PBS for 30 minutes. Fixed and permeabilized cells were incubated with monoclonal hybridoma supernatant harvested from 9E10 cells (myc epitope tag antibody) or with anti-GST antisera, washed in PBS, and then incubated with Cy3-conjugated secondary antibodies (Jackson

ImmunoResearch Laboratories, Inc.). After washing, the cover slips were mounted on glass slides with Prolong (Molecular Probes). The cells were viewed on a Noran OZ Laser Scanning Confocal Microscope and the data collected in a UNIX based Silicon Graphics INDY R5000 workstation or viewed on a Nikon deconvolution microscope. The figures were prepared using Photoshop 5.5 (Adobe) and the relative intensities have been adjusted to visualize spatial overlap.

Results

Ras interacting protein 69 (Rip69) was identified as a Ha-Ras interacting protein in a yeast two-hybrid screen of a mouse embryo cDNA library (14). Among the clones recovered in this screen were those that required an intact Ras effector domain for the Ras-Rip interaction. The proteins encoded by these isolates, which include Raf family members (14), the catalytic subunit of phosphatidylinositol 3-kinase (21), and guanine nucleotide exchange factors for Ral, were subsequently shown to be direct downstream targets of Ras. In addition, isolates were recovered that associated with Ras proteins mutant in the effector domain, among these was Rip69. The lack of a requirement for an intact Ras effector domain for the Ras-Rip69 protein-protein interaction suggests that the Rip69 isolate does not encode an effector of Ras function but is more likely to be either a regulator of Ras or a protein involved in the trafficking of Ras to the plasma membrane. Rip 69 encodes residues 20 to 186 of prenylated Rab acceptor protein PRA1.

Three published reports demonstrate that PRA1 interacts with members of the Rab family of GTPases, including Rab1, Rab3A, Rab 5 and Rab6 (15) (17) (22). Among small GTPases, PRA1 is thought to be a relatively specific partner for Rab proteins because, in two reports, PRA1 failed to interact with other members of the Ras superfamily, Ras, Rho and Rac (15) (17).

Because we had identified PRA1 in a two-hybrid screen with Ha-Ras, we investigated the ability of the full-length mouse PRA protein, PRA1, to interact with RhoA and TC21. In addition to interacting with Ha-Ras, PRA1 interacts with TC21 and RhoA, Figure 1. These results indicate that the interaction of PRA1 with small GTPases is not specific to Rab proteins; instead, PRA1 interacts with multiple members of the Ras superfamily, including Ha-Ras, RhoA and TC21. Although Rap1a does not interact with PRA1 in a yeast two-hybrid experiment, Rap1a does interact with PRA1 in vivo in mammalian cells (as described below and in Figure 2). Perhaps the LexA-Rap1a fusion protein is not prenylated efficiently in yeast or it may not localize to the nucleus as efficiently as the unprenylated form.

To confirm our yeast two hybrid experiments, we examined the association of Ha-Ras and RhoA with PRA1 in vivo. As shown in Figure 2A and B, Ha-Ras and PRA1 and RhoA and PRA1 associate in HEK293 cells overexpressing these proteins. In addition, because we did not observe an association of PRA1 with Rap1a in yeast, we examined the interaction in mammalian cells. As shown in Figure 2C, Rap1a and PRA1 co-associate in vivo in HEK293 cells. Thus, PRA1 associates with multiple members of the Ras superfamily in vivo.

Ha-Ras proteins undergo a series of post-translational modifications: 1) attachment of an isoprenoid farnesyl moiety to a cysteine located four residues from the carboxy terminus of the protein; 2) removal of the carboxy terminal tripeptide; 3) methylation of the carboxyl group of the farnesylated cysteine; and, 4) palmitoylation of two cysteines near the carboxy terminus of the protein, C-181 and C-184 (3) (4). To investigate the requirement for post-translational modification of Ha-Ras on the association of PRA1 with Ha-Ras, we examined the association of PRA1 with mutant Ras proteins missing key post-translational modifications, Figure 3. Ha-RasV12ΔCAAX is an activated Ras that lacks all post-translational modifications. Ha-

RasV12K6 is an activated Ras with polylysine substituted for S190, the last residue of the CAAX box; this mutant Ras is palmitoylated but not farnesylated (23). Ha-RasV12 S181, S184 is an activated Ras that is farnesylated, proteolyzed, and methylated, but not palmitoylated (23). PRA1 interacts with Ha-RasV12 S181, S184 but not Ha-RasV12 Δ CAAX or Ha-RasV12K6, Figure 3. Thus Ras proteins that are not palmitoylated are still capable of associating with PRA1, indicating that palmitoylation of Ras is not required for the association of the proteins. Ras proteins that are not farnesylated or are not modified in any way do not interact with PRA1, suggesting that farnesylated Ras is the binding partner for PRA1.

In order to determine whether the modified carboxy terminal tetrapeptide of Ha-Ras is both necessary and sufficient for recognition by PRA1, we assessed the association of PRA1 with GFP-CAAX, a GFP protein to which only the last 4 amino acids of Ha-Ras, CVLS, were added. This tetrapeptide is both necessary and sufficient for farnesyltransferase and geranylgeranyl transferase I to recognize and prenylate Protein A and Gi α , which have been mutated to end in a CAAX sequence (24) (25). Likewise, we expect that in yeast this tetrapeptide will be recognized and prenylated by yeast farnesyltransferase and likely proteolyzed and methylated because mammalian Ha-Ras when expressed in yeast is appropriately modified. As shown in Figure 4, PRA1 interacts with GFP-CAAX but not GFP. This result suggests that the molecular determinants for the binding of PRA1 reside within the CAAX box of Ha-Ras and that a modified tetrapeptide (farnesylated, proteolyzed and/or methylated) is both necessary and sufficient to mediate the protein-protein interaction.

The sole post-translational modification in common among the PRA1 interacting proteins, Rabs, Ras, Rho and TC21, is isoprenylation, the attachment of farnesyl or geranylgeranyl moieties to the proteins, Table 1. To demonstrate that post-translational

processing events subsequent to prenylation (proteolysis and/or methylation) are not essential for PRA1 binding, we examined the association of PRA1 with GFP-CVYS. GFP-CVYS contains a substitution of Y for L at position 187 in the CAAX box. A K-Ras 187Y mutant protein is prenylated but not further processed (33). As shown in Figure 4B, PRA1 interacts with GFP-CVYS. Taken together, our observations suggest that an isoprenoid moiety, either farnesyl or geranylgeranyl, is the critical recognition target for PRA1.

To confirm the prenylation dependency observed in yeast two hybrid experiments, modified and unmodified GST-Ha-Ras was purified from mammalian cells after Triton X-114 partitioning. GST-Ha-Ras from the detergent phase, modified Ras, interacted with PRA1 in vitro whereas GST-Ha-Ras from the aqueous phase, unmodified Ras, did not interact with PRA1 in vitro, Figure 5. Taken together, our observations in yeast and in vitro indicate that PRA1 preferentially associates with post-translationally modified (prenylated) Ras.

Sequences homologous to PRA1 are present in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, *Drosophila melanogaster*, and *C. elegans*. To determine if the association of PRA1 with small GTPases was conserved, we cloned the yeast homolog and assessed its ability to interact with Ha-Ras and RhoA. The yeast PRA1 protein, yPRA1, interacts with both Ha-Ras and RhoA, Figure 6. The association of Ha-Ras and yPRA1 is dependent on the post-translational modification of Ras because Ha-Ras Δ CAAX is unable to bind to yPRA1, Figure 6. Thus, the interaction of PRA1 with Ras family members is conserved in evolution.

Recent studies have demonstrated an association of Ha- and N-Ras with endomembranes (8) (26). Ha- and N-Ras are prenylated in the cytoplasm and then undergo additional modifications as they transit through the ER then Golgi. RhoA also is present in the ER and/or

endosomal vesicles (27) (28). The prenylation dependent association of small GTPases with PRA1, together with the ability of PRA1 to interact with small GTPases with effector domain mutations (a result which strongly suggests that PRA1 is not an effector of the small GTPases with which it interacts) suggested to us that PRA1 may function in an endomembrane compartment. Therefore, we compared the spatial distribution of PRA1 and Ha-Ras and RhoA in COS1 cells. Both Ha-Ras and PRA1 exhibit perinuclear staining, indicative of Golgi localization, and Ha-Ras and PRA1 co-localize in this perinuclear region, Figure 7A. In addition, RhoA and PRA1 also co-localize in the Golgi compartment, Figure 7B. Recent studies have shown that PRA1 is present in the Golgi compartment (16) and our studies using Golgi markers confirm this (data not shown).

The 15 carbon farnesyl and 20 carbon geranylgeranyl isoprenoid groups are hydrophobic, although the hydrophobicity of these groups do not support stable membrane localization. Since PRA1 specifically recognizes the isoprenoid moieties of small GTPases, the binding of PRA1 to prenylated small GTPases may facilitate their trafficking through the endomembrane system by masking the hydrophobicity of the isoprenoid groups.

Discussion

With this report, we demonstrate that PRA1 associates with multiple members of the Ras superfamily. We show that PRA1 associates with Ha-Ras, RhoA, TC21 and Rap1a. Others have shown that PRA1 associates with Rab family members (15-17). The sole feature in common among these small GTPases is the presence of farnesyl or geranylgeranyl isoprenoid moieties covalently linked to cysteines located at or near the carboxy terminus of the small GTPases

(Table 1). Our Ras CAAX box mutant studies (Figure 3), Triton-X114 partitioning studies (Figure 5), and GFP-CAAX/PRA1 protein-protein interaction studies (Figure 4) are consistent with the farnesyl or geranylgeranyl moiety of the small GTPase as being the key recognition target for PRA1.

Small GTPases are not the only proteins subject to modification by prenylation. Prelamin A, Lamin B, γ subunits of heterotrimeric G proteins, and serine/threonine kinases such as LKB1, are prenylated (3) (29). It will be interesting to determine whether PRA1 associates with proteins outside the Ras superfamily.

A search of the data bases reveals the presence of PRA1 homologs in many organisms. PRA1 homologs are present in both budding and fission yeast, in *A. thaliana*, *C. elegans*, *D. melanogaster* and *M. musculus*. yPRA1, the PRA1 homolog from budding yeast, interacts with multiple prenylated small GTPases (Figure 6). Thus, the interaction of PRA1 with multiple small GTPases is conserved in evolution and this conservation of the association suggests that the function of the PRA1-small GTPase interaction may also be conserved in evolution. Experiments are in progress to genetically decipher the physiological role of PRA1 in yeast.

The association of PRA1 with multiple small GTPases in vitro and in vivo suggests that PRA1 has a role that is held in common with all small GTPases that it interacts with. The feature in common among the small GTPases that PRA1 interacts with is their post-translational modification by farnesyl or geranylgeranyl isoprenoid moieties. We suggest that PRA1 binds prenylated GTPases to act as an escort protein for the GTPases. Biochemical fractionation and localization experiments show that PRA1 is found both in the cytoplasm and in the Golgi (16). PRA1 may associate with small GTPases after prenylation occurs in the cytoplasm to "solubilize" the hydrophobic prenyl motifs and thus facilitate trafficking through the

endomembrane system. Alternatively, PRA1 may function in the Golgi to facilitate the entry of small GTPases into vesicles for transport to cellular compartments. PRA1 is found in synaptic vesicle membranes and so PRA1 may cycle with the Ras proteins in the exocytic vesicles out to the plasma membrane (30).

PRA1 also associates with GDP dissociation inhibitor 1 (GDI1) (16). Addition of recombinant GDI1 to membranes prepared from PC12 cells was effective at removing Rab3A from the membrane; addition of PRA1 blocked the ability of GDI to extract Rab3A (16). These observations suggest that the opposing actions of GDI1 and PRA1 may influence the membrane localization of the Rab proteins. GDIs have been described that solubilize membrane-associated Rho proteins (31). Possibly the opposing action of GDI and PRA1 is a general mechanism for regulating the membrane status of small GTPases that are acted upon by GDIs.

The prevalence of Ras mutations in human tumors and the requirement of plasma membrane localization of Ras for cellular function suggested early on that inhibitors of farnesyltransferase (FTIs) might be a promising class of cancer therapeutics (5). Some of these FTIs are presently being evaluated in phase II clinical trials. However, small GTPases, such as RhoC which has been recently associated with promotion of metastasis (32), are geranylgeranylated and so inhibitors of geranylgeranyl transferase may also prove to be a promising class of cancer therapeutics. In addition, some GTPases are "switch-hitters," subject to either farnesylation or geranylgeranylation. So, inhibiting one pathway may not necessarily prove efficacious in cancer treatment. PRA1 binds both farnesylated and geranylgeranylated small GTPases and may act as a receptor or escort protein for Ras superfamily members. Thus, inhibitors directed against PRA1 may provide a means to pharmacologically intervene to inhibit

the signaling pathways activated aberrantly by both farnesylated and geranylgeranylated small GTPases, which can result in sustained cell growth (tumorigenesis) and metastasis.

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Figure Legends

Figure 1. PRA1 interacts with multiple members of the Ras superfamily of small GTPases. The interaction of small GTPases with PRA1 was assessed by yeast two hybrid analysis. The *S. cerevisiae* reporter strain L40 was transformed with a plasmid that expresses a Ras family member fused to the LexA DNA binding domain (pLex-Ras-V12, pLex-Rap1a-V12, pLexTC21-V23 or pLexRhoA-63L) and plasmids that express the VP16 activation domain alone (pVP16), the VP16 activation domain fused to the amino terminal domain of c-Raf (Rip51, positive control), the domain of PRA1 recovered in the yeast two-hybrid screen (Rip69), or full length mouse PRA1 (PRA1). Yeast transformants were grown overnight at 30°C in liquid culture maintaining selection for the plasmids (YC-WL) and dilutions of the overnight cultures were plated to YC-WL plates or to YC-WHULK plates to assess the activation of the *HIS3* reporter. A positive association between the LexA fusion protein and the VP16 fusion protein leads to growth in the absence of histidine (YC-WHULK plates). Activated Ras, TC21 and RhoA associate with PRA1 and Rip69.

Figure 2. Ha-Ras, RhoA and Rap1a interact with PRA1 in vivo. GST or GST fusion proteins were purified from lysates prepared from HEK293 cells transfected with the indicated constructs using glutathione sepharose. The pull-downs were subject to SDS-PAGE followed by western blotting with antibodies directed against the epitope tags on Ha-Ras, RhoA, or Rap1a (GST) or PRA1 (Myc).

A. GST-Ha-RasV12, but not GST or GST-Ha-RasV12ΔCAAX, associates with PRA1. B. GST-RhoA L63 but not GST associates with PRA1. C. GST-Rap1a but not GST associates with PRA1.

Figure 3. Farnesylation of Ha-Ras but not palmitoylation is required for the Ha-Ras/PRA1 association. The yeast L40 strain was transformed with the various plasmid combinations and the activation of the *HIS3* reporter assessed. pLex-Ha-RasV12ΔCAAX is a fusion of the LexA DNA binding domain to an activated Ras, which lacks the CAAX box; this mutant Ras is not post-translationally modified. pLex-Ha-RasV12K6 is a fusion of LexA to an activated Ras with polylysine substituted for S190, the last residue of the CAAX box; this mutant Ras is palmitoylated but not farnesylated. pLex-Ha-RasV12 S181, S184 is a fusion of LexA to an activated Ras that is farnesylated, proteolyzed, and methylated, but not palmitoylated. VP16-PRA1 is a fusion of the VP16 activation domain to full length PRA1. Rip51 expresses the amino terminal domain of c-Raf fused to VP16. PRA1 interacts with Ha-RasV12 S181, S184 but not HaRasV12ΔCAAX or Ha-RasV12K6. LexA-HaRasV12ΔCAAX and LexA-Ha-RasV12K6 are able to interact with Rip51 and thus lack of interaction of these mutant proteins with PRA1 is not simply due to lack of expression or inability to localize to the correct cellular compartment.

Figure 4. A. Addition of the Ha-Ras CAAX box to GFP is necessary and sufficient to mediate the association of PRA1 with GFP. B. Post-translational events subsequent to prenylation are not essential for PRA1 binding. The yeast L40 strain was transformed with the various plasmid combinations and the activation of the *HIS3* reporter assessed. pLexA-GFP CAAX expresses the LexA DNA binding domain fused to a green fluorescent protein (GFP) to which the carboxyl terminal Ha-Ras tetrapeptide CVLS has been added. pLexA-GFP expresses the LexA DNA binding domain fused to GFP. pLexA-GFP-CVYS expresses the LexA DNA binding domain fused to GFP to which a mutant Ha-Ras CAAX box, CVYS, has been added. LexA-GFP-CVYS

is prenylated but not further processed (methylated and/or proteolyzed). pVP16-yPRA1 expresses the VP16 activation domain fused to full length yeast PRA1. pVP16-PRA1 expresses the VP16 activation domain fused to full length mouse PRA1. pVP16, vector control. Both yeast and mouse PRA1 associate with GFP CAAX and with GFP-CVYS but not GFP.

Figure 5. Post-translationally processed Ha-Ras associates with PRA1.

GST-Ha-Ras or GST were transiently expressed in HEK293 cells and processed and unprocessed proteins prepared by a Triton X-114 partitioning assay. Hydrophobic proteins partition into the detergent phase, whereas hydrophilic proteins partition into the aqueous phase of Triton X-114. The processed Ha-Ras partitions into the detergent phase, whereas the unprocessed Ha-Ras is found in the aqueous phase. GST, a soluble protein, partitions exclusively into the aqueous phase. The GST fusion protein from equal proportions of each phase was captured on glutathione sepharose resin and mixed with VP16-Rip69 (amino acids 20 to 186 of PRA1 fused to VP16) or VP16 (control), prepared in reticulocyte lysates in the presence of 35-S methionine. The resin was washed after incubation to remove unbound proteins and then the proteins in each of the binding reactions were resolved by SDS-PAGE and subject to autoradiography (lower panel) followed by immunoblotting with anti-GST antisera and detected by enhanced chemiluminescence (upper panel). GST-Ha-Ras purified from the Triton X-114 detergent, but not aqueous, phase interacts with VP16-Rip69. GST does not associate with Rip69.

Figure 6. The association of PRA1 with Ha-Ras and RhoA is conserved in evolution. The yeast L40 strain was transformed with the various plasmid combinations and the activation of the *HIS3* reporter assessed. The yeast homolog of PRA1, yPRA1, associates with both Ha-Ras and

RhoA. The association of Ha-Ras with yPRA1 is observed only with the modified (Ha-RasV12) but not the unmodified (Ha-RasV12 Δ CAAX) Ras.

Figure 7. Subcellular localization of Ha-Ras V12, RhoA L63 and PRA1. Cos1 cells were transiently transfected with expression vectors for eGFP-PRA1 and either GST-RasV12 (A) or GST-RhoA-L63 (B). GST-Ha-RasV12 and GST-RhoA-L63 were detected by indirect immunofluorescence using a rabbit anti-GST primary antibody and CY3 secondary antibody. eGFP1-PRA1 was detected by epifluorescence. A similar localization of Ha-RasV12, RhoA-L63 and PRA1 to that shown here in fixed cells was observed when eGFP fusions to Ha-RasV12, RhoA-L63 and PRA1 were visualized in live cells (data not shown).

Table 1. PRA1 associates with multiple GTPases.
The sole feature in common is prenylation.

GTPase	C-terminal Sequence	Prenylation	AAX	Methylation	Palmitoylation	2-Hybrid interaction
Ha-Ras	DESGPGCMSCKCVLS	F	Y	Y	Y	++
TC21	TRKEKDKKGCHCVIF	F	Y	Y	Y	++
RhoA	LQARRGKKKSGCLVL	G	Y	Y	N	++
Rap1A	PVEKKKPKKKSCLLL	G	Y	Y	N	- ¹
Rab3a	QLSDQQVPPHQDCAC	G	N	Y	N	+*
Rab1	KIDSTPVKSASGGCC	G	N	N	N	+*

The carboxy terminal sequence of each GTPase is shown and the post-translational modifications each GTPase undergoes is indicated. F: Farnesylation.

G: geranylgeranylation. AAX: Proteolysis following prenylation. Y: yes. N: no.

2-Hybrid interaction: PRA1 + small GTPase. The two-hybrid interaction data for Ha-Ras, TC21, RhoA and Rap1a is derived from Figure 1 of this report and from published reports (15-17) for Rab3A and Rab1 (*). ¹Although Rap1a fails to interact with PRA1 in a yeast two hybrid test, Rap1a interacts with PRA1 in vivo in mammalian cells (see Figure 2 and text).

Figure 1

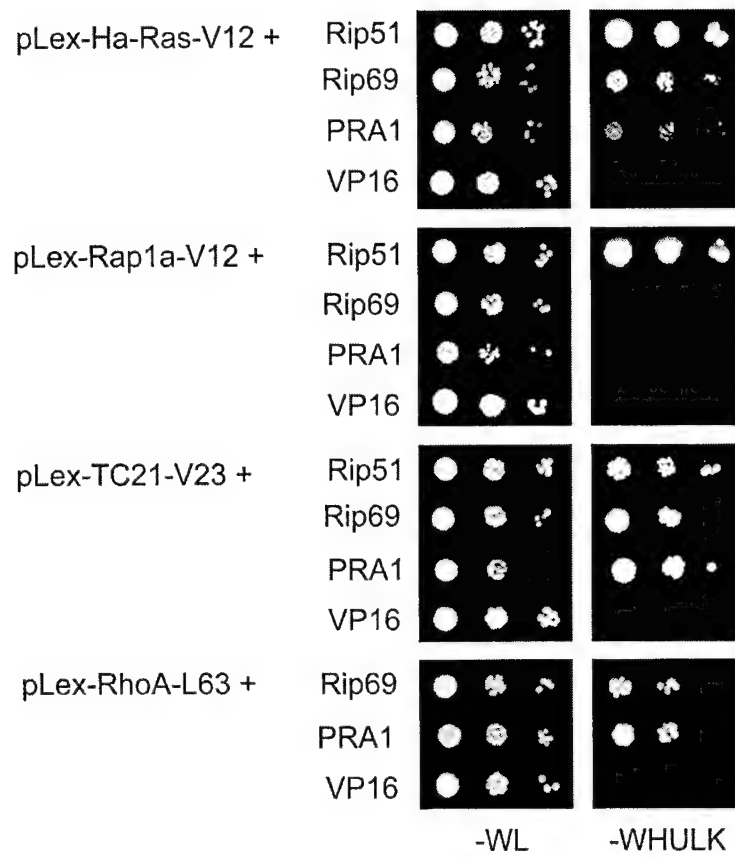
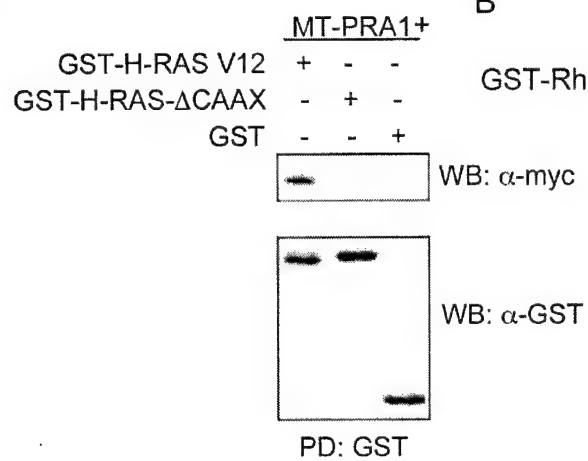


Figure 2

A



B

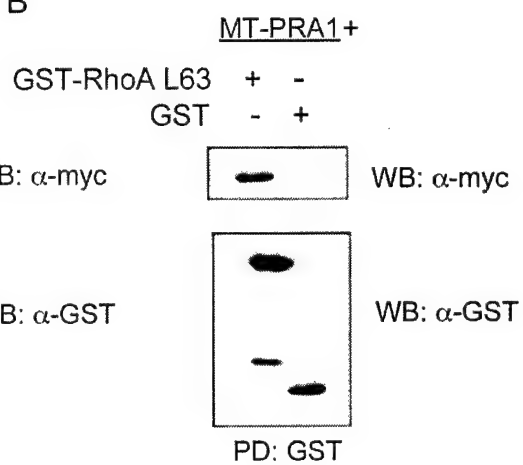


Figure 2C

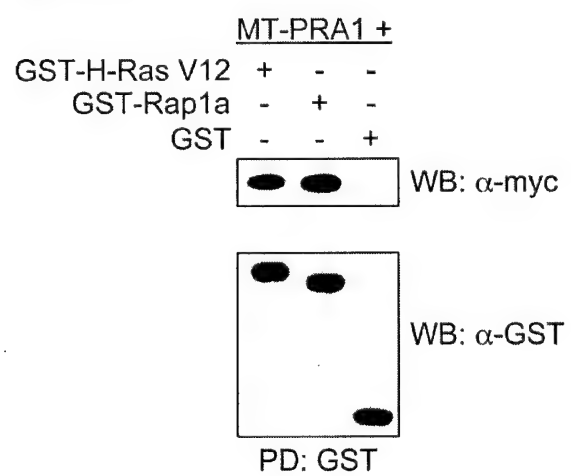
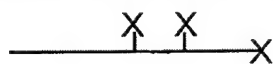
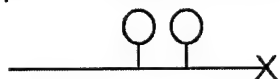


Figure 3

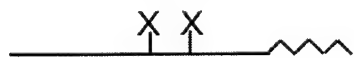
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pLex-H-RasV12K6 +



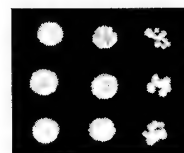
pLex-H-RasV12S181S184 +



PRA1

VP16

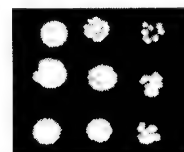
Rip51



PRA1

VP16

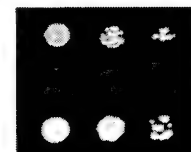
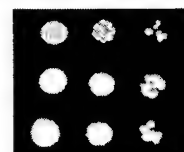
Rip51



PRA1

VP16

Rip51



-WL

-WHULK

Figure 4

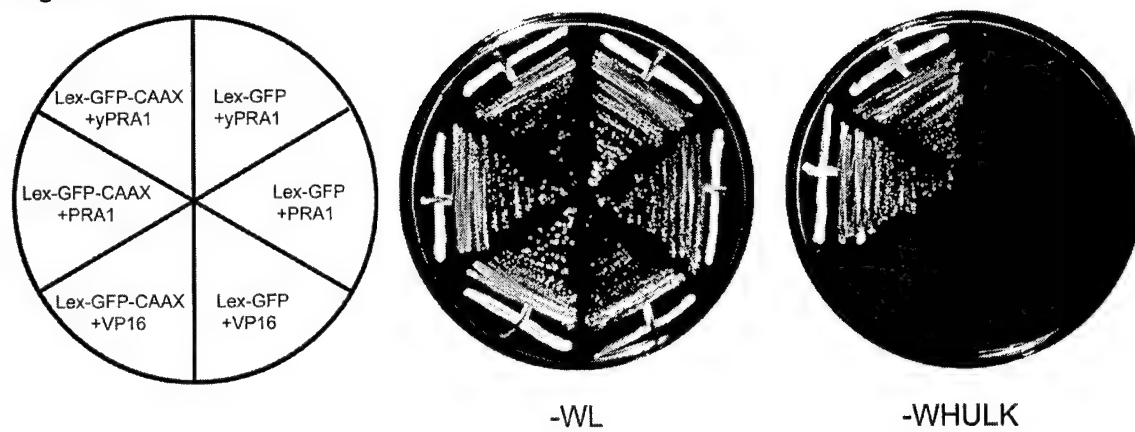


Figure 4B

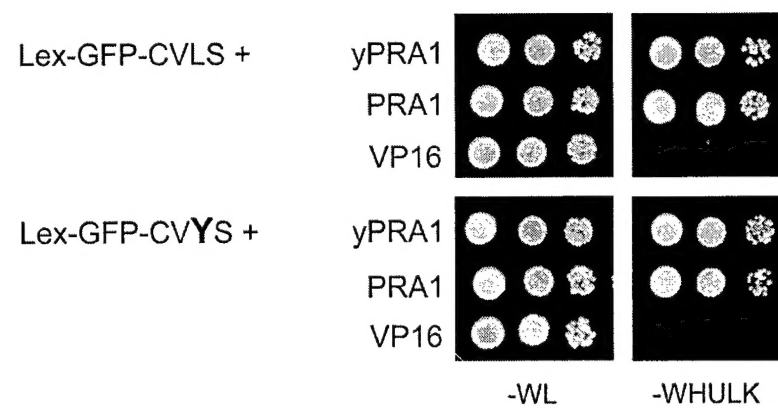


Figure 5

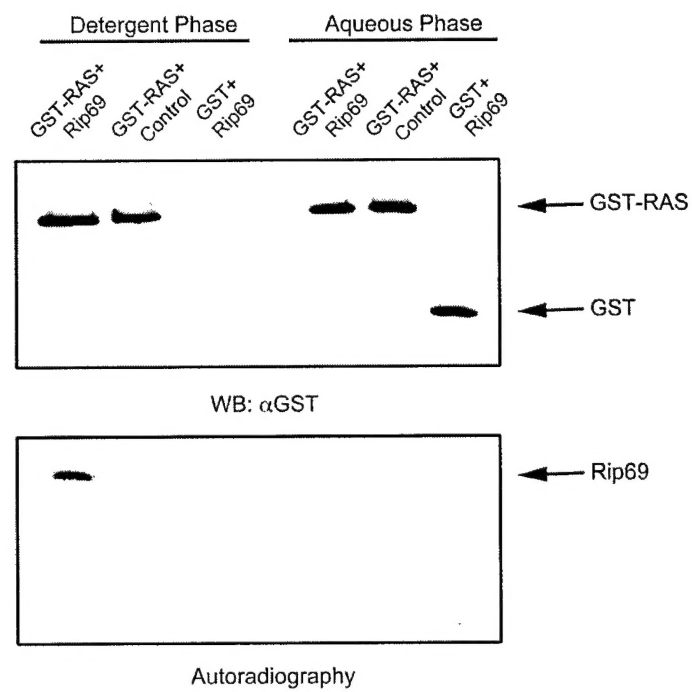


Figure 6

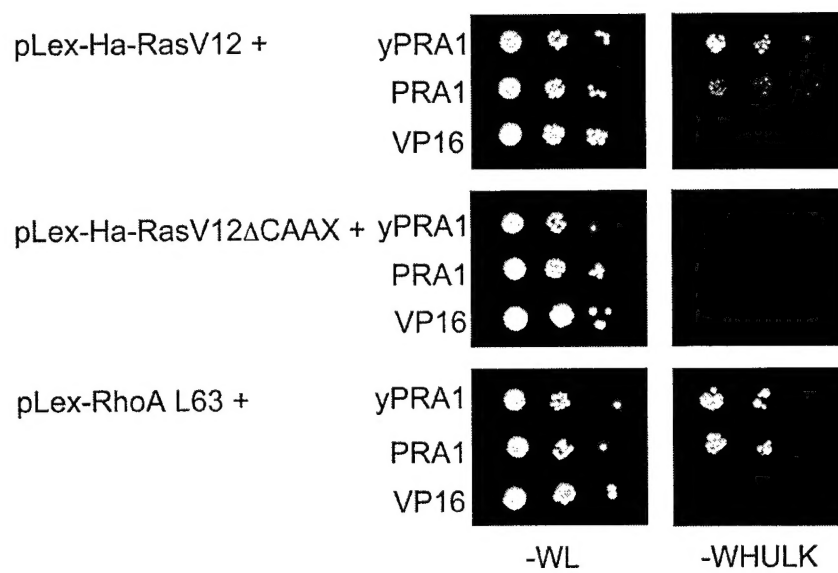


Figure 7

A



eGFP-PRA1

GST-Ha-RasV12

Overlap

B



eGFP-PRA1

GST-RhoA-L63

Overlap